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13. ABSTRACT (Maximum 200 Words) The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of PCa cells by methyl selenium (Se)/selenol. We hypothesized that methyl inhibits PI3K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. We have in the reporting period refined a methylselenol generation system based on methioninase with selenomethionine as substrate (Wang et al, Mol. Carcinogenesis, 2002) and studied the association of protein kinases and effects of PI3K inhibitors for apoptosis induction in DU145 cells by methyl Se and selenite (Jiang et al, Mol. Cancer Therapeutics, 2002). We compared the apoptosis responses of DU145 (androgen independent and mutant p53) and LNCaP (androgen dependent, wild type p53) PCa cell lines to methyl Se/selenol and to selenite. The LNCaP cells are PTEN mutant and possess high basal AKT activity and are more resistant to apoptosis induction by methyl Se, but was not cross resistant to cell death induction by selenite (AACR abstract, 2003). These studies lend additional credence to the role of PI3K-AKT in apoptosis signaling induction by the methyl Se pool. Furthermore, we have discovered a caspase-dependent apoptosis response induced by selenite in LNCaP cells, which is distinct from the lack of caspase involvement in DU145 cells after selenite exposure. This led to a hypothesis that p53 phosphorylation may play a crucial role in mediating apoptosis induced by a genotoxic Se agent through caspase-mediated execution (AACR Abstract, 2003). We will investigate additional cell lines for correlation between PI3K/AKT status and apoptosis sensitivity to methyl Se as well as p53 status and caspase-dependency for apoptosis induction by selenite in the next year. We will also initiate studies with transfections of mutant kinases/proteins to specifically address their roles in signaling pathways for apoptosis induction by selenium agents.				
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1. Introduction

The purpose of this research is to gain a better understanding of the biochemical pathways and molecular targets for the selective induction of apoptosis signaling and execution of PCa cells by methyl Se/selenol. We hypothesized that methyl Se/selenol inhibits PI₃K-AKT survival pathway leading to the activation of caspase-dependent apoptosis execution in PCa cells. The specific aims are to delineate the caspase-mediated execution pathways of apoptosis (Objective 1) and to critically test the role of PI₃K-AKT survival pathway in apoptosis signaling (Objective 2) induced by methyl Se/selenol.

We have in the current reporting period refined a methylselenol generation system based on methioninase with selenomethionine as substrate (Wang et al, Mol. Carcinogenesis, 2002, **appendix 1**) and studied the association of protein kinases and effects of PI3K inhibitors for apoptosis induction in DU145 cells by methyl Se and selenite (Jiang et al, Mol. Cancer Therapeutics, 2002, **appendix 2**). We compared the apoptosis responses of DU145 (mutant p53, androgen independent) and LNCaP (wild type p53, androgen dependent) PCa cell lines to methyl Se and to selenite. The LNCaP cells are PTEN mutant and possess high basal AKT activity and we have observed that they are more resistant to apoptosis induction by methyl Se, but was not cross resistant to cell death induction by selenite, which had been shown to cause DNA damage (AACR abstract ID number is 100956, 2003, **appendix 3**). These studies lend additional credence to the role of PI3K-AKT system in apoptosis signaling induction by the methyl Se pool. Furthermore, we have discovered a caspase-dependent apoptosis response induced by selenite in LNCaP cells, distinct from the lack of caspase involvement in DU145 cells after selenite exposure. This led to a hypothesis that p53 may play a crucial role in mediating apoptosis induced by a genotoxic Se agent through caspase-mediated execution (AACR Abstract ID number is 100969, 2003, **appendix 4**). Detailed report is as follows.

2. Key accomplishments

- 2.1 Induction of caspase-mediated apoptosis and cell cycle G₁ arrest by selenium metabolite methylselenol (*Molecular Carcinogenesis*, 2002) Appendix 1

Previous work based on mono-methyl selenium compounds that are putative precursors of methylselenol has strongly implicated this metabolite for inducing caspase-mediated apoptosis of human prostate carcinoma and leukemia cells and for inducing G₁ arrest in human vascular endothelial and cancer epithelial cells. To test the hypothesis that methylselenol itself was responsible for exerting these cellular effects, we examined the apoptotic action on DU145 human prostate cancer cells and G₁ arrest effect on human umbilical vein endothelial cells (HUVEC) of methylselenol generated with seleno-L-methionine as a substrate for L-methionine- α -deamino- γ -mercaptomethane lyase (EC4.4.1.11, also known as methioninase). Exposure of DU145 cells to methylselenol so generated in the sub-micromolar range led to caspase-mediated cleavage of poly(ADP-ribose)polymerase, nucleosomal DNA fragmentation and morphological apoptosis and resulted in a similar profile of biochemical effects as exemplified by the inhibition of phosphorylation of protein kinase B/AKT and extracellularly-regulated kinases 1/2 when compared to methylseleninic acid (MSeA) exposure. In HUVEC, methylselenol

exposure recapitulated the G₁ arrest action of MSeA on mitogen-stimulated G₁ progression during mid- to late-G₁ and this stage-specificity was mimicked by inhibitors of the phosphatidylinositol 3-kinase. Taken together, the results support methylselenol as an active selenium metabolite for inducing caspase-mediated apoptosis and cell cycle G₁ arrest. This **cell-free methylselenol generation system** is expected to have a significant utility for studying the biochemical and molecular targeting mechanisms of this critical metabolite and may constitute the basis of a novel therapeutic approach for cancer.

- 2.1 Distinct effects of methylseleninic acid vs. selenite on apoptosis, cell cycle and protein kinase pathways in DU145 human prostate cancer cells (*Molecular Cancer Therapeutics*, in press 2002). Appendix 2

While the anti-cancer mechanisms have not been clearly defined, one hypothesis relates to selenium metabolites, especially the mono-methyl selenium pool, generated under supranutritional selenium supplementation. To explore potential molecular targets for mediating the chemopreventive activity, we contrasted the effects of methylseleninic acid (MSeA), a novel precursor of methylselenol, vs. sodium selenite, a representative of the hydrogen selenide metabolite pool, on apoptosis execution, cell cycle distribution and selected protein kinases in DU145 human prostate cancer cells. Exposure of DU145 cells to 3 μ M MSeA led to a profound G₁ arrest at 24 h and exposure to greater concentrations led to not only G₁ arrest, but also to DNA fragmentation and caspase-mediated cleavage of poly(ADP-ribose)polymerase (PARP), two biochemical hallmarks of apoptosis. Immunoblot analyses indicated that G₁ arrest induced by the sub-apoptogenic doses of MSeA was associated with increased expression of P27kip1 and P21cip1, but apoptosis was accompanied by dose-dependent decreases of phosphorylation of protein kinase AKT and extracellular signal regulated kinase (ERK1/2) in the absence of any phosphorylation change in p38 mitogen activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK1/2). In contrast, selenite exposure caused S phase arrest and caspase-independent apoptotic DNA fragmentation, which were associated with decreased expression of P27kip1 and P21cip1 and increased phosphorylation of AKT/PKB, JNK1/2 and p38MAPK. Whereas apoptosis induction by MSeA exposure was not sensitive to superoxide dismutase added into the cell culture medium, cell detachment and DNA nucleosomal fragmentation induced by selenite exposure were greatly attenuated by this enzyme, supporting a chemical mediator role of superoxide for these processes. In spite of a temporal relationship of AKT and ERK1/2 dephosphorylation changes before the onset of PARP cleavage in MSeA-exposed cells, experiments with phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 did not show an enhancing effect of specific blocking of AKT on MSeA-induction of PARP cleavage. Taken together, exposure of DU145 cells to MSeA vs. selenite (under ample serum culture condition, i.e., 10%) induced differential patterns of cell cycle arrest and apoptosis execution as well as distinct patterns of effects on AKT, ERK1/2, JNK1/2 and P38MAPK phosphorylation and p27kip1 and p21cip1 expression. Multiple molecular pathways are likely differentially targeted by selenium metabolite pools to mediate cancer chemoprevention.

- 2.3 Refractoriness of LNCaP prostate cancer cells to methyl selenium induction of caspase-mediated apoptosis (AACR Abstract 100956, 2003) Appendix 3

We have shown that methylselenol and its precursor compounds induce caspase-mediated apoptosis resembling detachment-induced apoptosis "anoikis" in DU145 prostate cancer cells and have observed that cell death is associated with decreased phosphorylation of protein kinase AKT involved in cell survival signaling. Because DU145 cells contain wild type PTEN tumor suppressor gene, which negatively regulates phosphatidyl inositol-3-phosphate kinase(PI3K)-AKT pathway rendering low basal AKT activity in these cells, we tested whether mutant PTEN-bearing LNCaP prostate cancer cells with higher basal AKT activity were refractory to methyl selenium/selenol induction of apoptosis under ample serum culture condition. In dose-response experiments with 24 h exposure in the presence of 10% fetal bovine serum, LNCaP cells withstood much greater doses of methylseleninic acid as judged by cleavage of poly(ADP-ribose)polymerase and DNA fragmentation in comparison to DU145 cells. Similarly, methylselenol generated in the cell culture medium by methioninase using selenomethionine as a substrate failed to kill LNCaP cells at doses that efficiently killed DU145 cells. Nevertheless, LNCaP cells were more sensitive than DU145 cells to undergo apoptosis induced by sodium selenite exposure, which induces DNA single strand breaks. These results indicate that LNCaP cells are not cross-resistant to death signaling by selenium with a genotoxic mechanism(s). The role of PI3K-AKT in refractoriness to methyl Se exposure will be studied in coming years.

- 2.4 Selenite induces caspase-mediated apoptosis and p53 phosphorylation on serine-15 in LNCaP prostate cancer cells (AACR abstract 100969, 2003) Appendix 4

Our earlier work has shown that sodium selenite, a precursor of the hydrogen selenide metabolite pool induces DNA nucleosomal fragmentation and apoptosis of p53-mutant DU145 prostate cancer cells without caspase-mediated cleavage of poly(ADP-ribose)polymerase (PARP) (i.e., caspase-independent), whereas methylselenol and its precursors induce caspase-mediated apoptosis. Because selenite exposure is genotoxic through induction of DNA single strand breaks, and certain types of DNA damage induce p53-dependent apoptosis, we investigated whether selenite exposure of human LNCaP prostate cancer cells, which contain wild type functional p53 tumor suppressor gene, leads to caspase activation in a monolayer cell culture model. The results show that exposure of LNCaP cells for 24 h to lower micromolar concentrations of selenite led to dose-dependent DNA apoptotic fragmentation, procaspase activation and PARP cleavage. These changes were accompanied by p53 phosphorylation on Ser-15, but not on several other sites, and also by an induction of the expression of p53-target protein p21cip1 with no increase of total p53 content. A general caspase inhibitor zVADfmk and the specific inhibitors for caspase-8, 9 or 3 blocked PARP cleavage efficiently and decreased DNA fragmentation by a major extent. We are currently investigating whether the death signaling pathway from selenite exposure involves the following: selenite -> hydrogen selenide -> superoxide -> DNA single strand breaks -> p53 phosphorylation -> caspases.

3. Reportable outcomes

3.1 Peer reviewed Publications

Wang, Z. Jiang, C. and Lu JX. Induction of caspase-mediated apoptosis and cell cycle G₁ arrest by selenium metabolite methylselenol. *Mol Carcinogenesis*, 34: 113-120, 2002.

Jiang, C. Wang, Z., Ganther, H. and Lu, JX. Distinct effects of methylselenic acid vs. selenite on apoptosis, cell cycle and protein kinase pathways in DU145 prostate cancer cells. *Molecular Cancer Therapeutics*. In press, 2002.

3.2 Meeting abstracts

Junxuan Lu, Cheng Jiang. Refractoriness of LNCaP prostate cancer cells to methyl selenium induction of caspase-mediated apoptosis. Abstract No. 100956 American Association for Cancer Research 94th Annual meeting. Toronto, 2003.

Cheng Jiang, Junxuan Lu. Selenite induces caspase-mediated apoptosis and p53 phosphorylation on serine-15 in Incap prostate cancer cell. Abstract No. 100969 American Association for Cancer Research 94th Annual meeting. Toronto, 2003.

C. Jiang, Z. Wang and J. Lu. Selenium metabolite methylselenol inhibits G₁ to S progression of vascular endothelial cells by targeting phosphatidylinositol 3-kinase (PI3K) pathway during mid- to late-G₁ phase. American Association for Cancer Research 93rd Annual meeting. San Francisco, 2002.

J Lu, C. Jiang, Z. Wang, and H. Ganther. Selenium and cancer angioprevention: metabolite-specific anti-angiogenic attributes. Era of Hope 2002 DoD Breast Cancer Research Program Meeting September 25-28, 2002, at the Orange County Convention Center, Orlando, Florida. (Poster presentation).

3.3 Seminar presentations concerning selenium as cancer preventive agent by PI

March, 2002. University of Minnesota Hormel Institute, Austin, MN

March, 2002. University of Oklahoma School of Medicine, Oklahoma City, OK

Feb, 2002. University of Missouri, Columbia, MO.

Feb, 2002. University of Louisville, Louisville, KY.

Jan, 2002. North Carolina State Univ. School of Veterinary Medicine, Raleigh, NC

4. Conclusions

Work conducted during this current reporting period has further strengthened the differential pathways involved in signaling and executing apoptosis induced by different selenium metabolite pools. The specific role of PI3K-AKT pathway in methyl Se induced caspase-mediated death merits investigation under conditions of serum and oxygen deprivation conditions that exist prevalently inside growing lesions and cancers. Transfection of constitutive mutants into DU145 and LNCaP cells will be carried out in the next year to delineate their roles. The cell-free methylselenol system refined here will be useful to address efficacy of death induction in different cell lines without complication of metabolism. In addition, the role of p53 in mediating selenium-induced death should be investigated in reference to specific metabolite pools. The prevailing conclusion in the literature is that selenium induction of apoptosis is independent of p53 (1). That assertion merits further inquiry in light of our findings. As early lesions are more likely to retain p53 wild type function than full blown cancer cells, a p53-mediated caspase-dependent apoptosis induction by certain selenium metabolites may in part account for the observation of preferential sensitivity of early lesions to selenium intervention than cancer cells (2).

5. References

1. Kaeck M, Lu J, Strange R, Ip C, Ganther HE, Thompson HJ. Differential induction of growth arrest inducible genes by selenium compounds. *Biochem Pharmacol.* 1997 Apr 4;53(7):921-6.
2. Medina D, Shepherd FS. Selenium inhibition of the neoplastic transformation in preneoplastic mammary cell populations. *Cancer Lett.* 1984 Sep;24(2):227-34.

6. Appendices

1. Wang, Z. Jiang, C. and Lu JX. Induction of caspase-mediated apoptosis and cell cycle G₁ arrest by selenium metabolite methylselenol. *Mol Carcinogenesis*, 34: 113-120, 2002.
2. Jiang, C. Wang, Z., Ganther, H. and Lu, JX. Distinct effects of methylselenic acid vs. selenite on apoptosis, cell cycle and protein kinase pathways in DU145 prostate cancer cells. *Molecular Cancer Therapeutics*. In press, 2002.
3. Junxuan Lu, Cheng Jiang. Refractoriness of LNCaP prostate cancer cells to methyl selenium induction of caspase-mediated apoptosis. Abstract No. 100956 American Association for Cancer Research 94th Annual meeting. Toronto, 2003.
4. Cheng Jiang, Junxuan Lu. Selenite induces caspase-mediated apoptosis and p53 phosphorylation on serine-15 in Incap prostate cancer cell. Abstract No. 100969 American Association for Cancer Research 94th Annual meeting. Toronto, 2003.

BRIEF COMMUNICATION

Induction of Caspase-Mediated Apoptosis and Cell-Cycle G₁ Arrest by Selenium Metabolite Methylselenol

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Previous work based on mono-methyl selenium compounds that are putative precursors of methylselenol has strongly implicated this metabolite in the induction of caspase-mediated apoptosis of human prostate carcinoma and leukemia cells and G₁ arrest in human vascular endothelial and cancer epithelial cells. To test the hypothesis that methylselenol itself is responsible for exerting these cellular effects, we examined the apoptotic action on DU145 human prostate cancer cells and the G₁ arrest effect on the human umbilical vein endothelial cells (HUVECs) of methylselenol generated with seleno-L-methionine as a substrate for L-methionine- α -deamino- γ -mercaptomethane lyase (EC4.4.1.11, also known as methioninase). Exposure of DU145 cells to methylselenol so generated in the sub-micromolar range led to caspase-mediated cleavage of poly(ADP-ribose) polymerase, nucleosomal DNA fragmentation, and morphologic apoptosis and resulted in a profile of biochemical effects similar to that of methylseleninic acid (MSeA) exposure, as exemplified by the inhibition of phosphorylation of protein kinase AKT and extracellularly regulated kinases 1/2. In HUVEC, methylselenol exposure recapitulated the G₁ arrest action of MSeA in mitogen-stimulated G₁ progression during mid-G₁ to late G₁. This stage specificity was mimicked by inhibitors of phosphatidylinositol 3-kinase. The results support methylselenol as an active selenium metabolite for inducing caspase-mediated apoptosis and cell-cycle G₁ arrest. This cell-free methylselenol-generation system is expected to have significant usefulness for studying the biochemical and molecular targeting mechanisms of this critical metabolite and may constitute the basis of a novel therapeutic approach for cancer, using seleno-L-methionine as a prodrug.

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Key words: selenium; methylselenol; L-methionine- α , γ -lyase; seleno-L-methionine; apoptosis; G₁ arrest

INTRODUCTION

Recent human cancer prevention trials have indicated that selenium may be an effective chemopreventive agent for cancer that arises at many organ sites [1,2]. Methylselenol has been implicated for more than a decade as a critical *in vivo* selenium metabolite pool for anticancer activity [3–5]. Using mono-methyl selenium compounds that are putative precursors of methylselenol, we have identified several cellular, biochemical, and gene-expression responses that are distinct from those induced by selenium forms that enter the hydrogen selenide pool [6–9]. For example, methylselenocyanate and Se-methylselenocysteine induce apoptosis of mammary tumor epithelial cells without induction of DNA single-strand breaks [6–9]. On the other hand, the hydrogen selenide precursors sodium selenite and sodium selenide, for example, within an hour of exposure induce DNA single-strand breaks (i.e., genotoxic) and subsequent cell death by a combination of acute lysis and apoptosis [6–9].

We have reported that methylseleninic acid (MSeA), a novel methylselenol precursor, induces

DU145 human prostate carcinoma cell apoptosis through caspase-dependent execution [10]. Specifically, apoptosis induced by MSeA involves cell detachment, the activation of multiple caspases, mitochondrial release of cytochrome c, cleavage of poly(ADP-ribose) polymerase (PARP), and DNA nucleosomal fragmentation [10]. The last three actions require active caspases, as demonstrated with pharmacological inhibitors of these death proteases [10]. Independent of and complementing

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Abbreviations: MSeA, methylseleninic acid; PARP, poly(ADP-ribose) polymerase; HUVEC, human umbilical vein endothelial cell; PI3K, phosphatidylinositol 3-kinase; METase, L-methionine- α -deamino- γ -mercaptomethane lyase, also known as methioninase; SeMet, seleno-L-methionine; ECGS, endothelial cell growth supplement; AKT, protein kinase AKT; ERK, extracellularly regulated protein kinase.

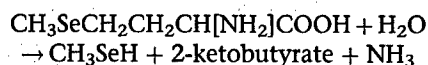
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our work with MSeA, Se-methylselenocysteine has been shown to cause caspase-dependent apoptosis of HL-60 human leukemia cells [11], which grow in suspension culture and do not require cell attachment for survival and mitogenesis. In contrast to methyl selenium compounds, selenite exposure induces apoptotic DNA fragmentation with minimal involvement of caspase-mediated execution in both prostate cancer and leukemia cell lines [10,11].

Furthermore, methyl selenium compounds have been shown to cause G₁ arrest in cancer epithelial cells [7–9] and in vascular endothelial cells [12], whereas selenite exposure induces S-phase arrest [7–9]. We have shown that MSeA exposure inhibits mitogen-stimulated human umbilical vein endothelial cell (HUVEC) G₁ progression to S phase, and the inhibitory activity appears to target a mechanism in mid-G₁ to late G₁ phase [12]. This stage-specific action can be mimicked by inhibitors of phosphatidylinositol 3-kinase (PI3K), suggesting a potential target pathway [12]. In a synchronized mouse mammary tumor cell culture model, exposure to MSeA or other methylselenol precursors during the mid-G₁ to late G₁ stage of cell-cycle progression was found to inhibit potently the progression to S phase [13]. These results suggest the possibility that methylselenol targets a critical mechanism of cell-cycle G₁-to-S progression despite the diverse nature of the mitogenic stimuli and signaling pathways in the different cell types [12,13].

Although the results obtained with various methyl selenium compounds have implicated methylselenol strongly as a common metabolite for the activities of G₁ arrest and apoptosis in diverse cell types, these studies have not been able to establish directly that methylselenol is responsible for inducing these cellular effects. In this communication, we provide experimental evidence that methylselenol generated in cell-culture medium by L-methionine- α -deamino- γ -mercaptomethane lyase (EC4.4.1.11, also known as methioninase or METase) using seleno-L-methionine (SeMet) as a substrate (by the following well-characterized reaction [14]) can recapitulate these cellular and biochemical effects:



MATERIALS AND METHODS

Chemicals and Reagents

MSeA (CH₃SeO₂H) was synthesized as described elsewhere [13,15] and was generously provided by Dr. Howard Ganther (University of Wisconsin-Madison). Intracellularly, MSeA most likely reacts with reduced glutathione to generate methylselenol (CH₃SeH) [13]. Bovine endothelial cell growth supplement (ECGS), heparin, SeMet, and L-methio-

nine were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies for total or cleaved PARP, total and phospho protein kinase AKT (AKT) and extracellularly regulated protein kinase (ERK1/2) were purchased from Cell Signaling Technology (Beverly, MA). [Methyl-³H]thymidine (20 Ci/mmol) was purchased from Du Pont-New England Nuclear (Wilmington, DE).

Methylselenol Generation

Purified METase of *Pseudomonas putida* (catalog no. 133-11933) and recombinant METase based on the gene from *Trichomonas vaginalis* produced in *Escherichia coli* (catalog no. 529-51731) were purchased from Wako Pure Chemical Industries, Ltd. (Richmond, VA). The enzyme preparations were reconstituted in phosphate-buffered saline shortly before use. For cell treatments, substrates for the enzyme (methionine or SeMet) were added first into the culture medium. Then the enzyme preparation was added directly into the cell-culture medium. Dose-response experiments were carried out with respect to METase activity and SeMet concentration, to establish conditions for comparing the morphologic and biochemical responses of cells to methylselenol versus MSeA.

Cell Lines and Culture Conditions

Cells were grown in humidified chambers at 37°C with 5% CO₂. DU145 human prostate cancer cells were cultured in RPM 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine, as described previously [10]. HUVECs were obtained from American Type Culture Collection (Manassas, VA) and were propagated in F12K medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 μ g/mL heparin, and 30 μ g/mL bovine ECGS, as described previously [12,15]. All cell-culture work was performed without antibiotics.

DU145 Apoptosis

Representative gross morphologic responses to selenium agents or other treatments were documented with a Polaroid camera at 200 \times magnification under a phase-contrast microscope [10]. DNA nucleosomal fragmentation as a marker of apoptosis was analyzed by gel electrophoresis as described earlier [10]. As an indicator of caspase-mediated apoptosis execution and for comparing biochemical action profiles elicited by methylselenol versus MSeA, we analyzed the cleavage status of PARP and the phosphorylation status of AKT and ERK1/2 by immunoblot analyses, as described elsewhere [10].

HUVEC G₁-S Progression Model

The ECGS-stimulated HUVEC cell-cycle progression model was used as we have described elsewhere [12]. In this model, it takes approximately 16 h from

the time of ECGS initial stimulation for cells to reach S phase, which peaks at 24 h [12]. Briefly, HUVECs were seeded in T₂₅ flasks in complete growth medium to 70–80% confluence and then were fed the described medium without ECGS for 48 h, to arrest cells in G₀. To compare the effects of methylselenol versus MSeA on ECGS-stimulated cell-cycle progression to S phase, [³H]thymidine (0.2 µCi/mL) and ECGS were added simultaneously with the selenium agents for 30 h. DNA synthetic activity was measured as cumulative [³H]thymidine incorporation into the trichloroacetic acid-precipitable fraction during 30 h of ECGS stimulation.

G₁-Stage-Specific Effect of Methylselenol

In earlier work we have shown that MSeA exerts its G₁ arresting activity after between 6 and 12 h of ECGS stimulation (mid-G₁ to late G₁), an effect that can be recapitulated by P13K inhibitors [12]. To determine whether methylselenol exerted a similar mid-G₁ to late G₁ action, 3 µM SeMet plus METase (~0.02 U/mL) was introduced either simultaneously with ECGS stimulation (lag time = 0) or 6 h (early-G₁ to mid-G₁), 12 h (late G₁), or 24 h (peak S phase) after ECGS stimulation had commenced (lag time = 6, 12, or 24 h). Ly294002 was dissolved in dimethylsulfoxide and used at a concentration of 25 µM following the same dosing schedule as for the selenium agents. The final dimethylsulfoxide concentration was below 0.1% by volume. The DNA synthetic activity was measured as [³H]thymidine cumulative incorporation into the trichloroacetic acid-precipitable fraction during 30 h of ECGS stimulation.

RESULTS

Gross Morphologic Responses Induced by Methylselenol Versus MSeA Exposure

In experiment 1, exposure of DU145 cells to 5 µM MSeA for 20 h induced cellular retraction into angular shapes (Figure 1A, panel f). A small proportion of the cells detached, displaying typical grapelike apoptotic bodies under phase-contrast microscopy. In the absence of METase, exposure to SeMet at up to 100 µM (which is approximately two orders of magnitude higher than the human plasma selenium level) did not cause cellular retraction and detachment, nor did this treatment induce visible morphologic features of apoptosis (Figure 1A, panel b). Similarly, METase plus its regular substrate L-methionine (Met) at 100 µM had no effect on these parameters (Figure 1A, panel e).

When METase (0.08 U/mL) was added into the medium with SeMet, as little as 1 µM SeMet induced morphologic responses similar to those of 5 µM MSeA exposure (Figure 1A, panel c vs. panel f), and a significant increase in morphologic features of apoptosis were apparent between 1 and 10 µM SeMet

(Figure 1A panels c and d). METase plus 100 µM SeMet led to rapid cell retraction and detachment within a few hours of exposure, and by 20 h only remnants of dead cells remained (not shown). These results indicated that methylselenol, not SeMet or the regular products of METase (i.e., methylthiol, 2-ketobutyrate, and NH₃) [14], accounted for induction of morphologic features of apoptosis under the conditions tested here.

Caspase-Mediated Apoptosis Induced by Methylselenol Versus MSeA Exposure

Earlier we showed that in DU145 cells PARP cleavage is a sensitive marker of caspase-mediated apoptosis induced by MSeA exposure [10]. In experiment 1, we analyzed PARP cleavage status to compare the efficacy of methylselenol with that of MSeA for inducing caspase-mediated apoptosis (Figure 1A, immunoblot). In accord with the lack of morphologic apoptosis responses, neither exposure to SeMet at up to 100 µM (lane b) nor exposure to METase with its regular substrate methionine at 100 µM (lane e) induced PARP cleavage. On the other hand, in the presence of METase (0.08 U/mL), methylselenol generated from 1 µM SeMet induced an extent of PARP cleavage similar to that of 5 µM MSeA exposure (lane c vs. lane f). The extensive PARP cleavage induced by methylselenol released from 10 µM SeMet (lane d) corroborated the massive morphologic apoptosis response (panel d). Compared with lane d, the decline in the extent of cleaved PARP in cells exposed to 100 µM SeMet plus METase suggested that other acute death execution mechanisms, in addition to caspase-mediated cleavage of PARP, probably were responsible for the rapid and massive death responses.

In subsequent experiments, we examined the dose responses of DU145 cells to SeMet initial concentrations and METase activity levels, to establish conditions that permitted a fair comparison of biochemical responses elicited by methylselenol versus MSeA. The K_m values of METase for Met (1.33 mM) and SeMet (0.51 mM) [14] are much higher than the concentrations of substrates in our experiments (i.e., 100 µM Met present in the cell-culture medium and up to 100 µM added SeMet). The velocity of methylselenol generation thereby is predicted to be an approximate linear function of the SeMet substrate concentration or the absolute amount of enzyme added, according to the Michaelis-Menten equation: $v = V_{max} * [substrate] / (K_m + [substrate]) \cong V_{max} * [substrate] / K_m$, when $K_m \gg [substrate]$.

As shown in Figure 1B, with 0.02 U/mL METase (experiment 2), methylselenol generated from 5 µM SeMet produced an extent of PARP cleavage similar to that of exposure to 5 µM MSeA, whereas with 0.1 U/mL METase (experiment 3), methylselenol produced from 1 µM SeMet was as effective as 5 µM

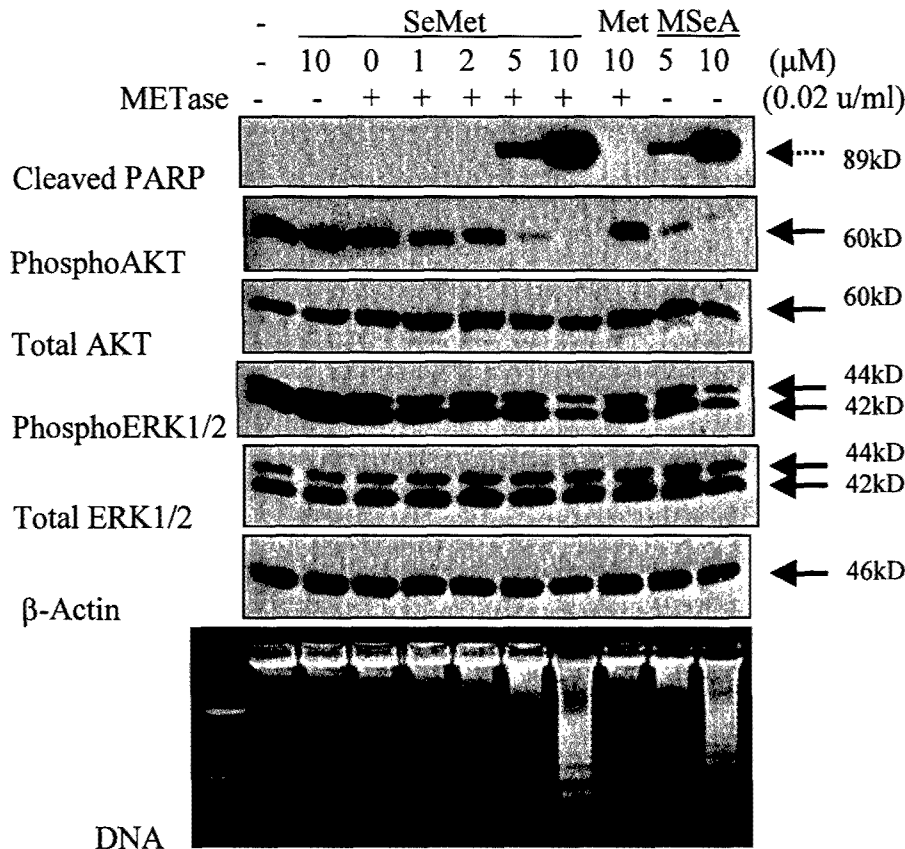


Figure 2. Immunoblot analyses of the effects of methylselenol versus MSeA on AKT and ERK1/2 phosphorylation status in DU145 cells after 20 h exposure and their relationship to apoptosis parameters. Apoptosis was assessed by detection of cleaved PARP (89 kD) and by DNA nucleosomal fragmentation. The level of total AKT or ERK1/2 remained unaffected by methyl selenium exposure. β -Actin was immunoblotted for gel-loading correction.

MSeA for inducing apoptosis. Because the enzyme preparations are expensive, it would be more economical to use a moderate yet physiologically relevant SeMet concentration with low METase levels to produce the desirable methylselenol generation velocity. In accord with this rationale, we determined that at an initial SeMet concentration of 10 μ M, ≥ 0.01 U/mL METase produced a robust apoptosis response, as detected by DNA nucleosomal fragmentation, whereas 0.001 U/mL was not sufficient and 0.005 U/mL was marginal (Figure 1C, experiments 4 and 5).

Effects of Methylselenol Versus MSeA Exposure on AKT and ERK1/2 Phosphorylation

Next we compared the phosphorylation status of these protein kinases to determine whether direct

methylselenol exposure produced biochemical action profiles similar to those of MSeA (Figure 2). We previously showed that MSeA-induced apoptosis in DU145 cells is associated with a decreased level of phosphorylation of AKT [10], an important survival kinase in many cell types [16]. As with MSeA exposure, methylselenol exposure decreased the phosphorylation level of AKT in a dose-dependent manner for the SeMet substrate and, to a lesser extent, that of ERK1/2, which have been known to mediate mitogenesis and/or survival in many cell types [17]. These inhibitory effects of methylselenol and MSeA appeared to be inversely proportional to the extent of PARP cleavage and DNA nucleosomal fragmentation that they induced (Figure 2). Neither methylselenol exposure nor MSeA exposure affected the total protein level of AKT or ERK1/2,

Figure 1. (A) Representative phase-contrast photomicrographs (200 \times) of DU145 human prostate cancer cells after 20 h of exposure to (a) phosphate-buffered saline as vehicle control, (b) 100 μ M SeMet alone, (c and d) 0.08 U/mL METase plus increasing levels of SeMet (c, 1 μ M; d, 10 μ M), (e and f) METase plus its regulate substrate Met at (e) 100 μ M or (f) 5 μ M MSeA. As a marker of caspase-mediated apoptosis, the PARP cleavage status of treated cells at 20 h of exposure was assessed by immunoblotting, using a primary antibody that recognized both the full-length and cleavage products. The solid

arrow points to intact PARP (115 kDa), and the dashed arrow points to cleavage product (89 kDa). Lanes corresponding to the picture panels are marked. (B) Apoptosis response (PARP cleavage) as a function of SeMet substrate concentration. Five times more METase was used in experiment 3 (0.1 U/mL) compared with experiment 2 (0.02 U/mL). (C) Apoptosis response (DNA fragmentation) as a function of METase activity level (experiment 4, range 0.02–0.2 U/mL; experiment 5, range 0.001–0.01 U/mL) while the initial SeMet concentration was held at 10 μ M.

indicating that the inhibitory effect of methylselenol on these protein kinases probably takes place through mechanisms that regulate phosphorylation (kinases, phosphatases) and their enzyme activities without changing the total number of molecules of the respective enzyme. These results show that methylselenol generated in the cell-culture medium induced caspase-mediated apoptosis markers and biochemical end points similar those of MSeA exposure.

Effects of Methylselenol Versus MSeA on ECGS-Stimulated HUVEC G_1 -to-S Progression

In an ECGS-stimulated G_0/G_1 entry and G_1/S progression model, our earlier work has shown that MSeA potentially inhibits mitogen-driven progression from G_1 to S phase [12]. In this model, exposure for 30 h to methylselenol inhibited ECGS-stimulated [3H]thymidine incorporation into DNA, with an IC_{50} of $\sim 1 \mu M$ for the SeMet substrate (Figure 3A).

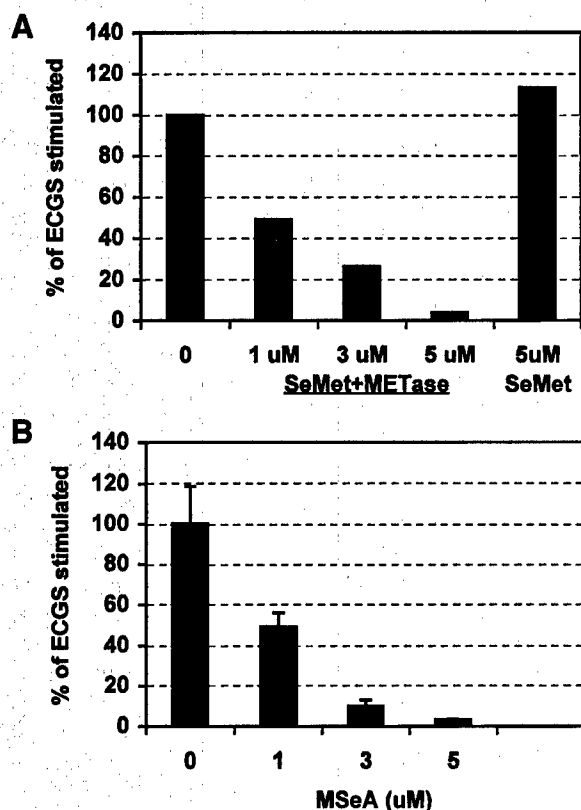


Figure 3. Dose-dependent inhibitory effects of enzymatically generated methylselenol (A) and MSeA (B) on ECGS-stimulated [3H]thymidine incorporation into HUVEC DNA. METase was ~ 0.02 U/mL. [3H]thymidine was added at time of ECGS stimulation for 30 h. The selenium treatments were commenced at the time of ECGS stimulation. ECGS-stimulated activity was set as 100%. The unstimulated basal incorporation value averaged $\sim 10\%$ of ECGS stimulation. (A) Each column represents the average of duplicate flasks with variation less than 10% of respective mean value. (B) Each column represents the mean of three independent experiments with duplicate flasks. The error bars indicate standard deviations of the respective mean values.

The dose-response pattern was identical to that of MSeA exposure (Figure 3B). As a negative control, SeMet without METase did not inhibit ECGS-stimulated DNA synthesis during 30 h of exposure (Figure 3A).

G_1 -Stage-Specific Action of Methylselenol Versus MSeA Exposure

We tested whether methylselenol also exerted the mid- G_1 to late G_1 stage-specific inhibitory action of MSeA, an effect that is mimicked by P13K inhibitors [12]. Methylselenol generated by METase plus $3 \mu M$ SeMet inhibited ECGS-stimulated progression to S phase by $\sim 94\%$ when the exposure was simultaneous with ECGS stimulation (lag time = 0) and inhibited by $\sim 93\%$ when the exposure was delayed for 6 h (early $-G_1$ to mid- G_1) (Figure 4, open bars). Even when exposure was started at 12 h of ECGS stimulation (late G_1), methylselenol inhibited S-phase entry by $\sim 60\%$. When the cells had entered peak S phase at 24 h, however, direct methylselenol exposure produced a minimal ($\sim 20\%$) inhibitory effect. The overall inhibitory pattern was nearly identical to that of MSeA exposure (Figure 4, filled bars) or a P13K inhibitor, LY294002 (Figure 4, striped bars). These results and data presented elsewhere [12] suggest that the antimitogenic action of methylselenol is exerted specifically at mid- G_1 to late G_1 phase (between 6 and 12 h), probably through a target pathway shared with P13K inhibitors.

DISCUSSION

The data presented here showed that direct exposure to enzymatically generated methylselenol recapitulated the apoptotic action of a methylselenol precursor compound, MSeA, in DU145 cells and its G_1 -arresting action in HUVECs. Specifically, direct methylselenol exposure of DU145 cells not only induced the same morphologic changes, PARP cleavage, and DNA nucleosomal fragmentation (Figure 1) but also exerted the same biochemical action profiles as did MSeA exposure, in terms of the phosphorylation status of AKT and ERK1/2 (Figure 2). Regarding the cell-cycle effect, methylselenol exposure, like MSeA exposure, appeared to target a control mechanism(s) in mid- G_1 to late G_1 (Figure 4). Because a P13K inhibitor, LY294002, could mimic the stage-specific effect of both methylselenol agents, P13K itself, or its downstream effector molecules, could be targeted specifically by methylselenol. Future efforts will be directed at identifying the molecular targets of methylselenol for regulating G_1 -to-S progression. The direct methylselenol generation system reported here is expected to have significant utility for studying such biochemical and molecular targeting mechanisms in cell-free assays and in cell-culture models.

The excellent efficacy of methylselenol for inducing DU145 cancer cell apoptosis and inhibiting

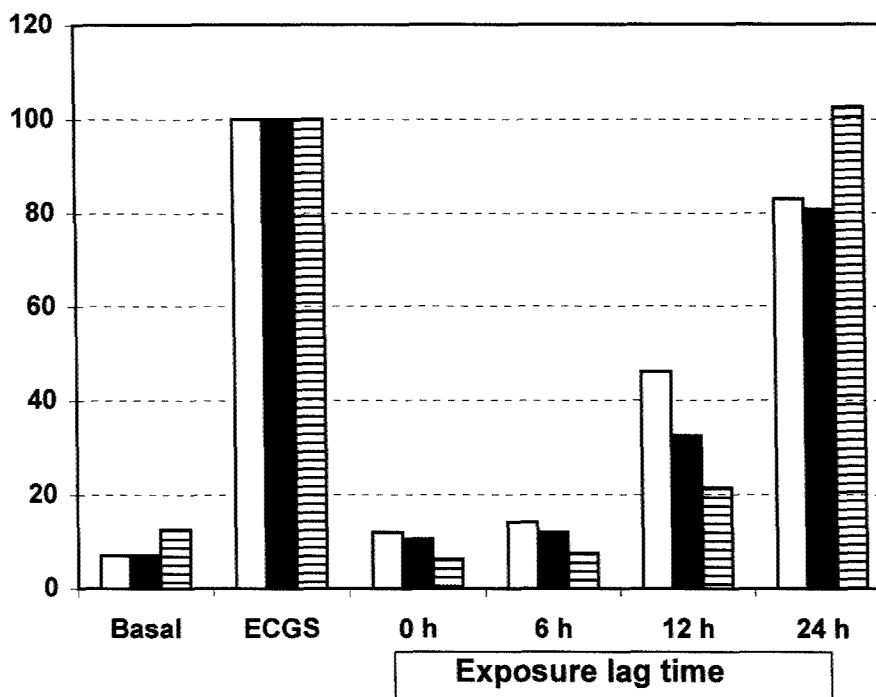


Figure 4. Cell cycle-stage effects of methylselenol generated by METase (~ 0.02 U/mL) with 3 μ M SeMet as substrate (open bars), 3 μ M MSeA (filled bars), 25 μ M PI3K inhibitor LY294002 (striped bars) on ECGS-stimulated [3 H]thymidine incorporation into HUVEC DNA during 30 h of ECGS stimulation. Methyl selenium or LY294002 treatment was either simultaneous with ECGS (lag time = 0 h) or

after ECGS stimulation had commenced for 6, 12, or 24 h, respectively (exposure lag time = 6, 12, or 24 h). ECGS-stimulated activity was set as 100%. Each column represents the average of duplicate flasks with variation less than 10% of the respective mean value.

ECGS-stimulated G₁-S progression is noteworthy in terms of its relevance to cancer chemoprevention and therapy. Whereas SeMet exposure as high as 100 μ M did not affect DU145 cell survival during the time frame of our experiments, methylselenol released from as little as 1 μ M SeMet in the presence of sufficient METase significantly induced caspase-mediated apoptosis of prostate cancer cells (Figure 1) and inhibited mitogen-stimulated HUVEC cell-cycle progression to S phase (Figure 3). These results suggest that a submicromolar amount of methylselenol should be sufficient to exert these actions. Although people who do not take selenium supplements usually have very little protein-free selenium in their serum [18], the level of nonprotein selenium metabolites is expected to increase sharply with chemopreventive or therapeutic use [19]. Under such conditions, the extra selenium that is not needed for selenoproteins is expected to enrich the methylselenol pool by the methylation pathway [20]. As a reference value, the mean plasma selenium concentration of subjects without selenium supplementation in recent human trials is ~ 1.5 μ M [1]. Supplementation (200 μ g/day as selenized yeast) that is associated with a greater than 50% reduction in the risk of prostate, colon, and lung cancers brings the mean selenium level to ~ 2.4 μ M [1]. It remains to be determined whether in vivo methylselenol can reach the submicromolar

levels, especially in the tumor local environment, that have the adverse impact on vascular endothelial cell proliferation and angiogenesis and cancer cell survival reported here.

Pertinent to the apoptotic action of methylselenol on cancer cells, it has been reported that in METase gene-transduced tumor cells, the cytotoxic activity of SeMet is increased 1000-fold compared with nontransduced cells in vitro [21]. Furthermore, SeMet treatment of nude mice bearing tumor cells expressing the METase transgene significantly inhibits ascites tumor growth and prolongs host survival [21], demonstrating a potential for METase gene therapy using SeMet as a prodrug substrate. The clinical utility of the methylselenol generation system described in this communication as a novel therapy, through intratumoral or tumor-targeted delivery of METase and either systemic or localized delivery of SeMet substrate, merits investigation.

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Distinct Effects of Methylseleninic Acid *versus* Selenite on Apoptosis, Cell Cycle, and Protein Kinase Pathways in DU145 Human Prostate Cancer Cells¹

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Abstract

Selenium has been implicated as a promising chemopreventive agent for prostate cancer. Whereas the anticancer mechanisms have not been clearly defined, one hypothesis relates to selenium metabolites, especially the monomethyl selenium pool, generated under supranutritional selenium supplementation. To explore potential molecular targets for mediating the chemopreventive activity, we contrasted the effects of methylseleninic acid (MSeA), a novel precursor of methylselenol, *versus* sodium selenite, a representative of the hydrogen selenide metabolite pool, on apoptosis execution, cell cycle distribution, and selected protein kinases in DU145 human prostate cancer cells. Exposure of DU145 cells to 3 μ M MSeA led to a profound G₁ arrest at 24 h, and exposure to greater concentrations led to not only G₁ arrest, but also to DNA fragmentation and caspase-mediated cleavage of poly(ADP-ribose) polymerase (PARP), two biochemical hallmarks of apoptosis. Immunoblot analyses indicated that G₁ arrest induced by the subapoptogenic doses of MSeA was associated with increased expression of p27kip1 and p21cip1, but apoptosis was accompanied by dose-dependent decreases of phosphorylation of protein kinase AKT and extracellular signal-regulated kinase (ERK1/2) in the absence of any phosphorylation change in p38 mitogen-activated protein kinase (p38MAPK) and c-Jun NH₂-terminal kinase (JNK1/2). In contrast, selenite exposure caused S-phase arrest and caspase-independent apoptotic DNA fragmentation, which were associated with decreased expression of p27kip1 and p21cip1 and increased phosphorylation of AKT, JNK1/2, and p38MAPK. Although apoptosis induction by MSeA exposure was not sensitive to superoxide dismutase added into the cell culture medium, cell detachment and DNA nucleosomal fragmentation induced by selenite exposure were greatly attenuated

by this enzyme, supporting a chemical mediator role of superoxide for these processes. Despite a temporal relationship of AKT and ERK1/2 de-phosphorylation changes before the onset of PARP cleavage in MSeA-exposed cells, experiments with phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 did not show an enhancing effect of specific blocking of AKT on MSeA-induction of PARP cleavage. Taken together, exposure of DU145 cells to MSeA *versus* selenite induced differential patterns of cell cycle arrest and apoptosis execution as well as distinct patterns of effects on AKT, ERK1/2, JNK1/2, and p38MAPK phosphorylation and p27kip1 and p21cip1 expression. Multiple molecular pathways are likely differentially targeted by selenium metabolite pools to mediate cancer chemoprevention.

Introduction

Human cancer prevention trials have shown an exciting promise of selenium supplement (approximately four times the recommended daily value of 55 μ g) as an effective chemopreventive agent for several major cancers including prostate, lung, and colon cancers in the United States (1) and liver cancer in China (2). However, the mechanisms underlying its cancer chemopreventive activity are poorly understood at the present. Studies using animal carcinogenesis models have strongly implicated a monomethyl selenium metabolite pool, perhaps methylselenol, as an active *in vivo* selenium species for the chemopreventive activity (3-5). We and others (6-10) have shown that methyl selenium compounds that are immediate precursors of methylselenol (e.g., MSeA⁴; Ref. 6; Fig. 1A) induce several biochemical and cellular responses that are distinct from those induced by selenium forms that initially enter the hydrogen selenide pool (e.g., sodium selenite; Fig. 1A), which can metabolically give rise to methylselenol *in vivo* by methylation (11). Distinguishing differences include the fact that the methylselenol precursors induce caspase-mediated apoptosis of DU145 prostate cancer cells (12) and HL-60 leukemia cells (13) without induction of DNA single-strand breaks (6-9, 13), whereas sodium selenite exposure induces DNA single-strand breaks (i.e., genotoxic; Refs. 6-9, 13) and apoptotic cell death with no involvement of caspases (12, 13). In mammary cancer cell lines (7-10) and vascular endothelial cells (14), exposure to methylselenol precursors arrests the cells in G₁ phase,

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⁴ The abbreviations used are: MSeA, methylseleninic acid; ERK, extracellular signal-regulated kinase; JNK, Jun NH₂-terminal kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PARP, poly(ADP-ribose) polymerase; SAPK, stress-activated protein kinase; SOD, superoxide dismutase.

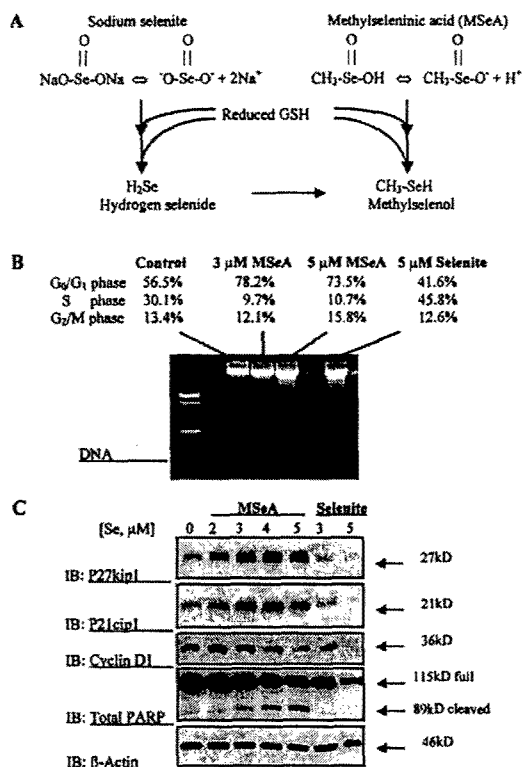


Fig. 1. A, chemical structure of selenite and MSeA and their likely proximal metabolites on reaction with reduced glutathione (11, 27). Dash arrow, limited conversion of hydrogen selenide to methylselenol by methylation in cell culture model. B, flow cytometric analyses of cell cycle distribution and electrophoretic analysis of apoptotic nucleosomal DNA fragmentation of MSeA- and selenite-treated DU145 cells at 24-h exposure. The percentage distribution data for each treatment group were the average of two flasks. The data were representative of three independent experiments. C, immunoblot detection of expression changes of cell cycle regulator proteins p27kip1, p21cip1, and cyclin D and of PARP cleavage as an indicator of caspase-mediated apoptosis execution in MSeA- and selenite-treated DU145 cells at 24-h exposure. β -Actin was probed to indicate evenness of loading of protein extract from each treatment. Arrows, proteins of interest. kD, M, in thousands.

whereas exposure to selenite arrests cells in S phase of the cell cycle (7–10). More recently, we have reported that methylselenol precursors inhibit the expression of vascular endothelial growth factor, an angiogenic cytokine, by cancer epithelial cells and inhibit the expression of matrix metalloproteinase-2 expression by vascular endothelial cells, and that these effects are absent for selenite exposure (15). These findings, taken together, support the presence of at least two selenium metabolite pools that induce distinct types of antiangiogenesis, apoptosis, and cell cycle responses. The molecular targets and pathways underlying these differential responses remain to be defined.

To explore potential molecular targets and pathways concerning prostate cancer chemoprevention and therapy with selenium, we focused the present work on selected protein kinases of cell proliferation and apoptosis signal transduction in DU145 human prostate cancer cells. This cell line represents an aggressive and metastatic prostate cancer

phenotype without functional p53 and retinoblastoma tumor suppressor proteins and is independent of androgen for growth and survival (16). In earlier work, we have used this model to delineate the caspase-mediated apoptosis execution pathways induced by MSeA exposure (12).

Several protein kinase pathways are known to regulate cell proliferation and survival. The PI3K pathway mediates cell cycle progression and cell survival through downstream effector proteins, one of which is the protein kinase AKT (17, 18). Inhibition of PI3K in some cell lines is sufficient to induce apoptosis, and ectopic expression of constitutively active mutants of PI3K or AKT inhibits apoptosis. Regarding selenium action, our earlier work has implicated an inhibition of the PI3K pathway activity as a likely mechanism for inhibiting cell cycle G₁ to S phase progression in vascular endothelial cells by either MSeA (14) or methylselenol (19). The significance of PI3K/AKT pathway as a potential target for methyl selenium-induced cell cycle arrest and apoptosis signaling in prostate cancer cells is not known.

In addition to the PI3K-AKT pathway, a family of closely related serine-threonine protein kinases, known as MAPKs, have been implicated in cell proliferation and apoptosis signaling in diverse model systems (20–22). Mammalian MAPKs include three broad families: the classic ERKs (ERK1/2), JNKs (also termed SAPK1), and p38MAPK (also known as SAPK2a). In general, ERK1/2 are activated by mitogens and survival factors (20–22), leading to cell cycle entry and progression through modulating expression of G₁ cyclins and the activity of cyclin-dependent kinases, which are, in part, regulated by the cyclin-dependent kinase-inhibitory proteins p27kip1 and p21cip1 (23, 24). JNK1/2 and p38MAPK are stimulated by stress and apoptotic signals; and, in many instances, either or both mediate cell death signaling (21, 22). Therefore, the balance of signals among the various protein kinase pathways may be critical for cell proliferation and apoptosis (25). To our knowledge, there had been no extensive published work on the PI3K-AKT and the various MAPK pathways in relationship to selenium induction of prostate cancer apoptosis and cell cycle arrest.

In this report, we document distinct effects of MSeA versus selenite exposure on the phosphorylation states of AKT, ERK1/2, JNK1/2, and p38MAPK and the expression of p27kip1 and p21cip1 in DU145 prostate cancer cells and their associations with distinct patterns of cell cycle arrest and apoptosis execution. We present data supporting a differential involvement of superoxide generation for apoptosis induction by the two forms of selenium in DU145 cells. Furthermore, we show results from experiments with PI3K inhibitors to probe the significance of the PI3K-AKT pathway for methyl selenium induction of apoptosis signaling.

Materials and Methods

Chemicals and Reagents. Sodium selenite pentahydrate was purchased from J. T. Baker, Inc. (Phillipsburg, NJ). MSeA ($\text{CH}_3\text{SeO}_2\text{H}$) was synthesized as described elsewhere (26, 27). Intracellularly, MSeA most likely reacts with reduced glutathione to generate methylselenol CH_3SeH (27). Bovine liver catalase and bovine erythrocyte (Cu, Zn)-SOD, the PI3K

inhibitor wortmannin, and an antibody for β -actin were purchased from Sigma Chemical Co. (St. Louis, MO). LY294002 was purchased from CalBiochem-Novabiochem Corp (La Jolla, CA). Antibodies for full-length human PARP or the cleaved PARP (M_r 89,000 fragment), caspase-7 and those for protein kinases and their phosphorylated forms (AKT Ser473; ERK1/2 Thr202/Tyr204; JNK Thr183/Tyr185; p38MAPK Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA).

Cell Culture and Treatments. DU145 cells were originally obtained from the American Type Culture Collection. DU145 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics as described previously (12). Cells at 50–70% confluence, usually 24–48 h after plating, were given a medium change and treated with selenium or other agents. To standardize selenium exposure, cells were treated in a medium volume to culture surface area ratio of 0.2 ml/cm² (15 ml for a T75 flask and 5 ml for a T25 flask). Concentrated selenium stock solutions were diluted in PBS to 1-mM working solutions before the addition to culture medium. In experiments in which PI3K inhibitors wortmannin and LY294002 were used, these compounds were dissolved in DMSO as concentrated stocks. After necessary dilution, the inhibitor(s) and MSeA were mixed into treatment media first and then fed to cells. DMSO (2 μ l/ml or less) was added to groups that did not receive the inhibitor to control for solvent vehicle effects. DMSO at the concentration used did not by itself cause any observable adverse morphological responses.

Cell Cycle Distribution and Apoptosis Evaluation. After experimental treatments, spent media were collected and spun at 1000 \times g for 5 min to collect detached cells or floaters, which were combined with respective adherent cells for cell cycle distribution analyses by flow cytometry at the University of Colorado Cancer Center flow cytometry core facility, Denver, CO. For the detection of DNA nucleosomal fragmentation as a biochemical indicator of apoptosis, DNA was extracted from adherent cells and floaters combined (unless stated otherwise) and analyzed by agarose gel electrophoresis as described previously (28). Cleavages of PARP and caspase-7 as markers of caspase-mediated apoptosis were detected by immunoblot analyses (12).

Immunoblot Analyses. After experimental treatments for the designated duration, DU145 cell lysates (floaters and adherent cells combined) were prepared as described previously (12). Supernatants after centrifugation (14,000 \times g for 20 min) were recovered, and the protein content was quantified by the Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). Twenty to 40 μ g of total protein were size-separated by electrophoresis on SDS-polyacrylamide gels under nonreducing conditions. The proteins were electroblotted onto nitrocellulose membranes and probed for the phosphorylated and/or the total forms of each kinase. For the detection of PARP and caspase cleavages, 80–100 μ g of total protein extract were used per treatment. Positive control samples obtained from the antibody suppliers were used whenever available.

Results

Distinct Cell Cycle Arrest and Cell Death Responses Elicited by MSeA versus Selenite Exposure. Flow cytometric analyses of cell cycle distribution of DU145 cells that had been exposed to 3 or 5 μ M MSeA for 24 h showed an \sim 2/3 reduction of S-phase cells and an enrichment of G₁ phase cells without significant changes of G₂-M cells (Fig. 1B). At 3 μ M or lower concentration of exposure, G₁ arrest was achieved without apoptosis, as indicated by lack of nucleosomal DNA fragmentation (Fig. 1B) and caspase-mediated PARP cleavage (Fig. 1C). In time-course experiments, the G₁ arrest effects began to manifest at \sim 12 h of exposure to MSeA (not shown).

In contrast to the G₁ arrest effect observed in MSeA exposed cells, cells exposed to 5 μ M selenite were arrested in S phase without any significant effect on G₂-M cells (Fig. 1B). Exposure to 3 μ M selenite did not affect the distribution pattern in comparison with the control cells (not shown). The S-phase arrest induced by selenite exposure was associated with DNA nucleosomal fragmentation (Fig. 1B) with no involvement of PARP cleavage (Fig. 1C).

Differential Effects of MSeA versus Selenite Exposure on Cell Cycle Regulatory Proteins. Biochemically, G₁ versus S-phase arrest effects induced by these two types of selenium were differentially associated with the expression patterns of p27kip1 and p21cip1, two key inhibitory proteins for cyclin-dependent kinases for regulating G₁ cell cycle progression in many cell types (23, 24). After 24-h exposure to MSeA, a dose-dependent increase of p27kip1 protein level, starting with as little as 2 μ M MSeA, was evident (Fig. 1C). p21cip1 protein level showed a similar pattern of increase, albeit the magnitude of change was less dramatic (Fig. 1C). In contrast to the expression patterns in MSeA exposed cells, a reduction of p27kip1 and p21cip1 expression level was observed in cells exposed to 5 μ M selenite (Fig. 1C). The expression level of cyclin D1, an important G₁ cyclin for cell cycle progression (24), was decreased in cells exposed to an apoptogenic level of either MSeA or selenite (Fig. 1C). These data indicate that elevated expression levels of p27kip1 and p21cip1 proteins were associated with G₁ arrest in MSeA-exposed cells irrespective of apoptosis and that the decreased expression of these two proteins was associated with S-phase arrest and apoptosis induced by selenite exposure.

Differential Involvement of Superoxide in MSeA versus Selenite Induction of Apoptosis. As far as primary chemical mediators of apoptosis signaling induced by the two forms of selenium are concerned, our earlier work has implicated superoxide for the genotoxic and apoptotic actions of selenite in mouse leukemia cells using a SOD-mimetic agent (9). This hypothesis was further strengthened by a recent study in LNCaP prostate cancer cells with a different SOD mimetic agent that effectively blocked the apoptotic efficacy of selenite exposure (29).

In the present study, we assessed the impact of SOD (200 units/ml) and catalase (1000 units/ml) added to cell culture media on apoptosis induced by MSeA or selenite at 20-h exposure. The enzyme combination by itself did not affect cell morphology (Fig. 2A, a' versus a) or DNA nucleosomal

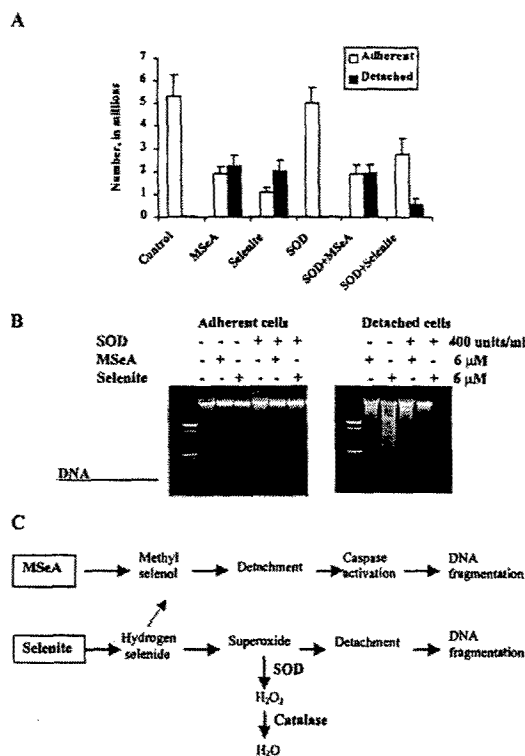


Fig. 3. A, effects of SOD (400 units/ml) coinubation on MSeA- versus selenite-induced cell detachment after 24 h of exposure. The concentration of each selenium agent was 6 μ M in this experiment. Conditioned media were collected and spun to pellet the detached cells. Adherent cells were trypsinized off T75 cell culture flasks. The number of adherent cells and detached cells was counted manually with a hemocytometer. Vertical bar, SD of replicate counting of 5–10 fields per sample. The data were representative of three similar experiments. B, DNA nucleosomal fragmentation status of MSeA- versus selenite-exposed adherent cells and detached cells from the experiment in A. C, schema for involvement of superoxide in apoptosis induction pathways in DU145 cells by MSeA versus selenite exposure.

In contrast to the suppression effects of MSeA exposure on AKT phosphorylation, exposure to selenite for 24 h at both a subapoptotic (3- μ M) and apoptotic dose (5- μ M) increased the phosphorylation level of AKT (Fig. 4). Contrary to the lack of effects by MSeA exposure on JNK1/2 or p38MAPK phosphorylation, selenite exposure at 5 μ M led to increased phosphorylation of these stress- and apoptosis-related protein kinases (Fig. 4). There was no appreciable selenite effect on the phosphorylation level of ERK2, although a minor inhibitory action on the phosphorylation of ERK1 might be associated with the apoptotic dose of selenite (Fig. 4). These results, therefore, confirm and extend the differential associations of these major protein kinases with apoptosis (12) and cell cycle arrest of DU145 prostate cancer cells induced by the two types of selenium.

Temporal Patterns of MSeA-induced Phosphorylation Changes of Protein Kinases. To probe the connection among protein kinase phosphorylation status and apoptosis induced by MSeA exposure, we next investigated the temporal patterns of AKT and ERK1/2 changes and p27kip1

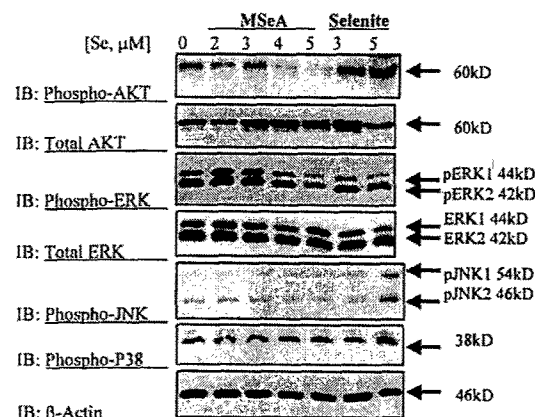


Fig. 4. Effects of MSeA versus selenite exposure for 24 h on the phosphorylation status and expression level of selected protein kinases in a dose-response experiment (same as in Fig. 1C). β -Actin expression was probed to indicate evenness of loading of protein extract from each treatment. Arrows, proteins of interest.

expression in relationship to apoptosis execution in DU145 cells (12 h and beyond; Fig. 5A). Exposure to 5 μ M MSeA led to an accelerated PARP cleavage between 12 and 16 h. Hypophosphorylation of AKT as well as of ERK1/2 was detected at 12 h. p27kip1 expression was elevated in the same time frame as the accelerated cleavage of PARP. The phosphorylation status of p38MAPK was not affected during the exposure period examined.

In an experiment designed to examine the more immediate responses (4–12 h; Fig. 5B), MSeA exposure increased AKT phosphorylation between 4 h and 8 h and then led to a sharp decrease of AKT phosphorylation at 10 h, which preceded the occurrence of accelerated PARP cleavage at 12 h. In contrast to AKT, the phosphorylation level of ERK1/2 was not increased during the acute MSeA exposure phase (up to 8 h). A reduction of ERK1/2 phosphorylation level was observed at 10 h of exposure, coinciding with the sudden switching of AKT phosphorylation status between 8 h and 10 h. These time course experiments indicated that the dephosphorylation of AKT and ERK1/2 preceded the occurrence of caspase-mediated apoptosis execution and of the onset of increased expression of p27kip1 and further ruled out an involvement of p38MAPK phosphorylation in cell cycle arrest and apoptosis execution induced by MSeA.

Minimal Contribution of AKT Phosphorylation Changes to MSeA Induction of Apoptosis. The temporal patterns of AKT and ERK phosphorylation changes in relation to caspase-mediated apoptosis suggested potential contributions to apoptosis signaling induced by MSeA exposure. Because of the known significance of PI3K/AKT pathway for supporting cell survival in other models (17, 18), we next examined the interaction of MSeA with PI3K inhibitors. As shown in Fig. 6A, treatment of DU145 cells with wortmannin for 4 h and 20 h significantly inhibited AKT phosphorylation but did not, by itself, lead to apoptosis. Wortmannin cotreatment with MSeA did not significantly alter the extent of PARP cleavage at 20 h when compared with that of MSeA exposure alone.

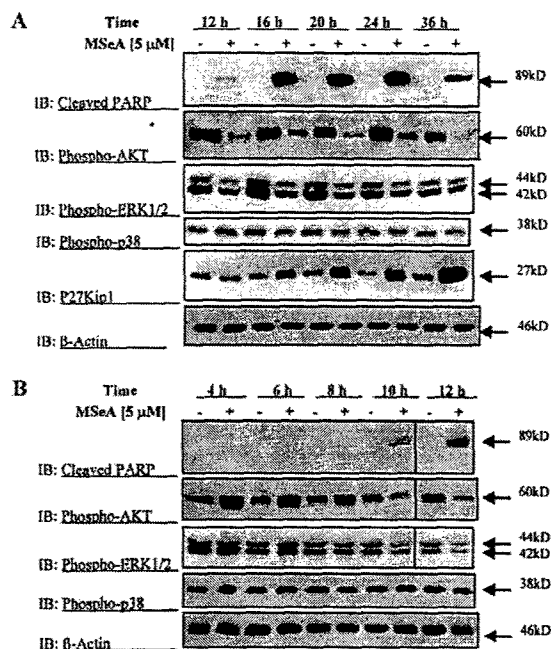


Fig. 5. Time course (A, 12–36 h; B, 4–12 h) of effects of MSeA exposure on protein kinase phosphorylation status and expression of p27kip1 in relationship to MSeA-induced cleavage of PARP in DU145 cells. These two experiments were done several months apart, which may account for the slight different timing of onset of PARP cleavage.

Because of a concern over the instability of wortmannin in aqueous solution (30), we then examined the effect of a more stable PI3K inhibitor LY294002 (31). LY294002 was introduced at 8 h of MSeA exposure, a time frame just before AKT undertaking a dramatic switch from hyper- to hypophosphorylation. Although LY294002 specifically inhibited AKT phosphorylation without affecting ERK1/2 phosphorylation, its cotreatment with MSeA exposure did not increase the extent of caspase-mediated PARP cleavage over that of MSeA exposure alone (Fig. 6B). Taken together, these results indicated that MSeA induction of AKT dephosphorylation might be one of many contributory signals for apoptosis signaling, but, by itself, it is not sufficient to account for apoptosis induction in this cell line.

Discussion

Prostate cancer is the most common histological malignancy and second leading cause of cancer deaths among North American men (32). Prostate cancer chemoprevention with dietary supplements holds strong promise for reducing the risk of this deadly disease. The prostate appears to be a responsive organ site for the cancer chemopreventive activity of selenium in American men (1). Although the normal prostate epithelial cells *in vivo* have very slow growth kinetics, increased mitogenic activity, cell cycle dysregulation, and increased cell survival as a result of autocrine and paracrine activation loops are common features of malignant prostate cancer cells (16, 33, 34). Antiproliferative activity through cell cycle arrest and induction of apoptosis are ex-

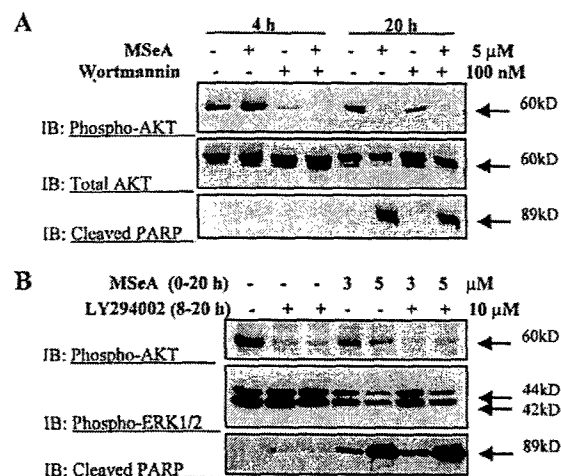


Fig. 6. *A*, effect of PI3K inhibitor wortmannin on AKT phosphorylation and MSEA-induced PARP cleavage in DU145 cells. *B*, effect of PI3K inhibitor LY294002 introduced 8 h after initiation of MSEA exposure on AKT and ERK1/2 phosphorylation and PARP cleavage at 20 h in DU145 cells. Arrows, proteins of interest.

pected to contribute to the direct actions of selenium on target epithelial cells to inhibit the growth of prostate cancerous lesions in the context of cancer chemoprevention. The present work was undertaken within the framework of selenium metabolites and their potential molecular targets for mediating cell cycle arrest and apoptosis.

We documented and characterized the differential effects of MSeA and selenite in DU145 prostate cancer cells on apoptosis signaling and cell cycle arrest with respect to PI3K/AKT, ERK1/2, p38MAPK, and JNK1/2 kinases. Pertaining to cell cycle action, MSeA exposure led to a profound G₁ arrest, irrespective of apoptosis induction; in contrast, selenite exposure led to cell cycle arrest in S phase (Fig. 1B). These patterns were in agreement with data obtained in mammary cancer cell lines (7–10). The cell cycle arrest effects were associated with differential patterns of expression of p21cip1 and p27kip1 (Fig. 1C), in that MSeA-arrested cells contained much elevated p27kip1 and p21cip1, whereas these proteins were down-regulated in the selenite-exposed cells. In a time course experiment, p27kip1 induction by exposure to an apoptogenic level of MSeA occurred concomitantly with PARP cleavage (Fig. 5A). Because cell cycle G₁ arrest and p27kip1 and p21cip1 induction occurred at subapoptotic doses of MSeA exposure, it will be important in future work to determine whether G₁ arrest is a prerequisite for apoptosis signaling, and what role P27/kip1 and p21cip1 play in methyl selenium induction of G₁ arrest.

Concerning the potential chemical mediators of apoptosis signaling induced by these two types of selenium, experiments with SOD and catalase indicated that selenite induction of caspase-independent apoptosis was sensitive to and inhibited by the addition of SOD in the culture media, but not catalase alone (Figs. 2 and 3). Furthermore, the data indicated that cell detachment and DNA fragmentation within the floaters, but not cytoplasmic vacuole formation in the adher-

ent cells (Fig. 2A), were SOD-sensitive cellular events with selenite exposure (Fig. 3). These data together support superoxide generation as a primary mediator for death signaling induced by selenite exposure. Because cytoplasmic vacuole formation precedes cell detachment on selenite exposure, its significance for death signaling remains to be established in light of the recent finding that cytoplasmic vacuoles represent swollen mitochondria in LNCaP prostate cancer cells (29). Likewise, the relationships among superoxide generation, DNA single-strand breaks, and caspase-independent apoptosis execution require further investigation.

Our data did not show a significant effect of SOD/catalase or SOD alone on MSeA induction of caspase-mediated apoptosis (Figs. 2 and 3), indicating that MSeA action was not likely mediated by either superoxide or hydrogen peroxide. However, it has been reported that intracellular methylselenol generated by methionine- γ -lyase using selenomethionine as a substrate leads to massive apoptosis and production of superoxide (35). However, the measurement of superoxide production in that study was done with a selenomethionine substrate level at least a magnitude higher than that necessary to induce apoptotic response (35). The discrepancy between our results and this cited work may relate to the vastly different levels of cell apoptosis in the two studies or to the MSeA exposure in our own, which may produce methylselenol as well as additional selenium metabolites that contribute to apoptosis signaling. More work will be needed to clarify this issue.

With respect to protein kinase pathways and selenium induction of apoptosis signaling and cell cycle arrest, a number of points were noteworthy. First, MSeA exposure led to changes of AKT and ERK phosphorylation without affecting JNK1/2 or p38MAPK, whereas selenite exposure led to increased phosphorylation of AKT, JNK1/2, and p38MAPK (Fig. 4). Second, these changes in protein kinase phosphorylation were observed in closer association with apoptosis than with cell cycle arrests (Figs. 1 and 4). The latter observation cast doubt on the hypothesis that phosphorylation/dephosphorylation of these protein kinases serves as primary signaling for the cell cycle-inhibitory action of either selenium in this cell line. However, it should be recognized that the possibility exists for selenium to modulate the activities of protein kinases and other enzymes through phosphorylation-independent mechanisms such as redox modification (36, 37).

To further explore the connection between AKT and ERK phosphorylation changes and MSeA-induced apoptosis signaling, we examined the time course of their changes in relationship to caspase-mediated apoptosis execution. The results (Fig. 5) provided a rough temporal sequence of events during MSeA induction of apoptosis. Exposure to MSeA led to an acute elevation of AKT phosphorylation that lasted through 8 h. Because no enhancing effect by MSeA was observed for ERK1/2 or p38MAPK or JNK1/2 within the same time frame, the acute hyperphosphorylation action by MSeA exposure is likely specific for AKT itself or for the PI3K-AKT pathway. The onset of caspase-mediated apoptosis execution as revealed by the appearance of cleaved

PARP was observed several hours after the sudden onset of dephosphorylation of AKT and ERK1/2. These temporal patterns suggested the potential significance of either one or both protein kinase pathways for apoptosis signaling induced by MSeA. The data from experiments with PI3K inhibitors (Fig. 6) showed that the specific inhibition of the PI3K-AKT axis alone in this cell line was not sufficient to trigger apoptosis within the time frame studied. It is, therefore, likely that MSeA induction of dephosphorylation of AKT is only one of many contributory signals, but not in itself sufficient, for apoptosis. Further work is needed to dissect the contribution of the PI3K-AKT and ERK1/2 pathways to apoptosis signaling using genetic approaches with constitutively active mutants of these pathway constituents.

In summary, the data showed distinct patterns of modulation of PI3K-AKT, ERK1/2, JNK1/2, and p38MAPK by MSeA versus selenite exposure, in close association with apoptosis execution through caspase-dependent and -independent pathways, respectively. These differences, plus the distinct cell cycle actions and differential involvement of superoxide as a chemical mediator of apoptosis, further contrasted the actions of two selenium metabolite pools in the DU145 prostate cancer cell line. An understanding of the biochemical and molecular details for these distinct actions of the two pools of selenium will be important for designing more effective selenium agents to specifically target desirable molecular pathways and cellular processes for prostate cancer prevention.

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Abstract # 100956

To be presented at the
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REFRACTORINESS OF LNCAP PROSTATE CANCER CELLS TO METHYL
SELENIUM INDUCTION OF CASPASE-MEDIATED APOPTOSIS

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Methylselenol has been implicated as an active selenium metabolite pool for cancer chemopreventive effect, at least in part, through its apoptogenic activity under supranutritional selenium supplementation. We have shown that methylselenol and its precursor compounds induce caspase-mediated apoptosis resembling detachment-induced apoptosis "anoikis" in DU145 prostate cancer cells and have observed that cell death is associated with decreased phosphorylation of protein kinase AKT involved in cell survival signaling. Because DU145 cells contain wild type PTEN tumor suppressor gene, which negatively regulates phosphatidylinositol-3-phosphate kinase (PI3K)-AKT pathway rendering low basal AKT activity in these cells, we tested whether mutant PTEN-bearing LNCaP prostate cancer cells with higher basal AKT activity were refractory to methyl selenium/selenol induction of apoptosis. In dose-response experiments with 24 h exposure in the presence of 10% fetal bovine serum, LNCaP cells withstood much greater doses of methylseleninic acid as judged by cleavage of poly(ADP-ribose) polymerase and DNA fragmentation in comparison to DU145 cells. Similarly, methylselenol generated in the cell culture medium by methioninase using selenomethionine as a substrate failed to kill LNCaP cells at doses that efficiently killed DU145 cells. Nevertheless, LNCaP cells were more sensitive than DU145 cells to undergo apoptosis induced by sodium selenite exposure, which induces DNA single strand breaks. These results indicate that LNCaP cells are not cross-resistant to death signaling by selenium with a genotoxic mechanism(s). The role of PI3K/AKT in conferring refractoriness against methylselenium is being tested by transfection of constitutively active mutants of AKT and other approaches. This work was supported by grants from the Department of Defense Prostate Cancer Research Program and the National Cancer Institute to JL.

Abstract # 100969

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**SELENITE INDUCES CASPASE-MEDIATED APOPTOSIS AND P53
PHOSPHORYLATION ON SERINE-15 IN LNCAP PROSTATE CANCER CELLS**

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The cancer chemopreventive effect of selenium may in part be related to the apoptogenic activity of its metabolites. Our earlier work has shown that sodium selenite, a precursor of the hydrogen selenide metabolite pool induces DNA nucleosomal fragmentation and apoptosis of p53-mutant DU145 prostate cancer cells without caspase-mediated cleavage of poly(ADP-ribose)polymerase (PARP) (i.e., caspase-independent), whereas methylselenol and its precursors induce caspase-mediated apoptosis. Because selenite exposure is genotoxic through induction of DNA single strand breaks, and certain types of DNA damage induce p53-dependent apoptosis, we investigated whether selenite exposure of human LNCaP prostate cancer cells, which contain wild type functional p53 tumor suppressor gene, leads to caspase activation in a monolayer cell culture model. The results show that exposure of LNCaP cells for 24 h to lower micromolar concentrations of selenite led to dose-dependent DNA apoptotic fragmentation, procaspase activation and PARP cleavage. These changes were accompanied by p53 phosphorylation on Ser-15, but not on several other sites, and also by an induction of the expression of p53-target protein p21cip1 with no increase of total p53 content. A general caspase inhibitor zVADfmk and the specific inhibitors for caspase-8, 9 or 3 blocked PARP cleavage efficiently and decreased DNA fragmentation by a major extent. We are currently investigating whether the death signaling pathway from selenite exposure involves the following: selenite -> hydrogen selenide -> superoxide -> DNA single strand breaks -> p53 phosphorylation -> caspases. This work was supported by grants from the Department of Defense Prostate Cancer Research Program and the National Cancer Institute to JL.